

Oral gene delivery with chitosan–DNA nanoparticles generates immunologic protection in a murine model of peanut allergy

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Food allergy is a common and often fatal disease with no effective treatment. We describe here a new immunoprophylactic strategy using oral allergen-gene immunization to modulate peanut antigen-induced murine anaphylactic responses. Oral administration of DNA nanoparticles synthesized by complexing plasmid DNA with chitosan, a natural biocompatible polysaccharide, resulted in transduced gene expression in the intestinal epithelium. Mice receiving nanoparticles containing a dominant peanut allergen gene (pCMVArah2) produced secretory IgA and serum IgG2a. Compared with non-immunized mice or mice treated with 'naked' DNA, mice immunized with nanoparticles showed a substantial reduction in allergen-induced anaphylaxis associated with reduced levels of IgE, plasma histamine and vascular leakage. These results demonstrate that oral allergen-gene immunization with chitosan–DNA nanoparticles is effective in modulating murine anaphylactic responses, and indicate its prophylactic utility in treating food allergy.

Food allergy poses a considerable public health problem, especially in children, because of the potential severity of allergic hypersensitivity. In the US, food allergies are the most common cause of anaphylaxis treated in hospital emergencies^{1,2}. About 100 fatal cases of food-induced anaphylaxis occur in the US each year³, with peanuts and tree nuts together representing the leading cause of fatal and near-fatal reactions^{1,3,4}. Most food-allergic responses involve immunoglobulin E (IgE)-mediated reactions², and failure to develop, or a breakdown in, oral tolerance may result in the production of food-specific IgE antibodies. IgE and allergens activate mast cells and basophils through the high-affinity IgE receptor. This activation causes the release of histamine and other mediators, leading to systemic anaphylactic reactions³.

Although peanut allergy is an increasingly important public health problem, its only proven treatment consists of educating patients to completely avoid all possible sources of peanuts. However, many accidental exposures (up to 50% of patients per year^{5,6}) occur because of the ubiquitous use of peanut protein in a variety of food products. Two small clinical trials in which escalating doses of allergen extracts were administered within a period of days demonstrated only limited efficacy and unacceptable side effects. Given the large number of patients with potentially fatal food allergy, the extreme difficulty in avoiding all food allergen exposure and the lack of efficacy for standard immunotherapy, effective prophylactic and therapeutic strategies are urgently needed.

DNA vaccination studies to modulate aero-allergen-induced IgE synthesis and airway hyper-responsiveness have produced promising results^{9–11}. Intramuscular or intradermal administration of 'naked' plasmid DNA encoding an antigen results in synthesis of

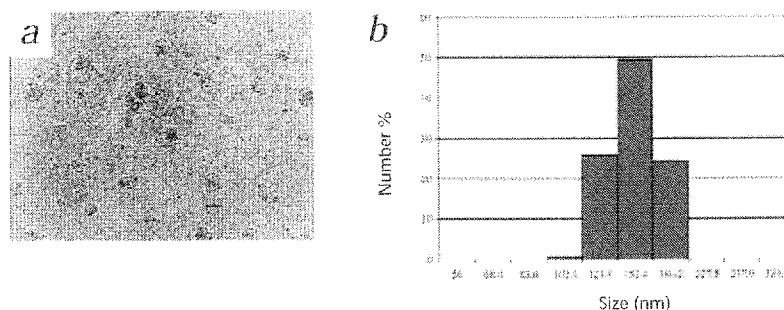
the antigen protein by the cells and subsequent development of both cellular and humoral immune responses to the antigen^{9,10,12–15}. However, despite the recent success of DNA-based immunization through intramuscular, intradermal and subcutaneous routes, oral vaccination with 'naked' DNA has been mostly ineffective¹⁶. Oral vaccination is useful not only because it generates mucosal immunity but also because of its high patient compliance, ease of administration and applicability for mass vaccination especially in third-world countries. Complex coacervates of DNA and chitosan¹⁷, a natural polysaccharide available from crustacean shells, could be used as a delivery vehicle in gene therapy and vaccine design. Chitosan is a biocompatible and slowly biodegradable polymer that has been widely used in controlled drug delivery^{18–23}, and it may provide a less immunogenic and non-toxic carrier for successful oral delivery of plasmid DNA. Chitosan also increases the transcellular and paracellular transport across mucosal epithelium²⁴, further indicative of its potential in oral gene delivery and in generating protective mucosal immune responses. Here we report oral immunization using chitosan–DNA nanoparticles carrying the gene for a principal peanut allergen, Arah2, and demonstrate their efficacy in modulating antigen-induced hypersensitivity in a murine model of peanut allergy.

Nanoparticle synthesis and characterization

We synthesized the nanoparticles by complexing high-molecular-weight (about 390,000 Da) chitosan with plasmid DNA. We obtained uniform particles by adding 0.02% chitosan, pH 5.7, at 55 °C to plasmid DNA (50 µg/ml in 50 mM sodium sulfate) during high-speed vortexing. Transmission and scanning electron microscopy showed that freshly prepared particles are approximately 150–300 nm in size and fairly spherical (Fig 1a). The

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Fig. 1 Appearance and size distribution of DNA nanoparticles. **a**, Transmission electron micrograph of chitosan-DNA nanoparticles. Scale bar represents 210 nm. **b**, Size distribution of freshly prepared nanoparticles. Size was measured using photon correlation spectroscopy (dynamic light scattering) and data were plotted as number distribution.



plasmid is partially protected from DNase degradation in this formulation²⁵ and its gel migration properties are unchanged by the process of forming complexes (results not shown). Dynamic light scattering measurements showed a unimodal particle size distribution (number average) between 100 and 200 nm (Fig. 1b). The zeta potential was approximately +10 mV at pH 5.7 and close to neutral at pH 7. Thus, the particles are probably positively charged at gastric and early duodenal pH but neutral thereafter at more physiological or alkaline pH.

Gene expression studies

To assess the expression and distribution of transduced genes after oral DNA delivery, we fed AKR/J mice either chitosan-DNA nanoparticles containing the LacZ gene (p43LacZ) or 'naked' plasmid DNA (p43LacZ). We determined the tissue expression of bacterial β -galactosidase (LacZ) in the stomach and small intestine 5 days after the oral administration (Fig. 2a-d). The stained sections represent, on average, 10% of the whole small intestine. Although naive mice and mice fed 'naked' DNA showed some background staining, mice fed the nanoparticles showed a higher level of gene expression in both the stomach and the small intestine. We counterstained frozen sections of the whole tissue from Fig. 2a with nuclear-fast red (Fig. 2e). Histologically, it seems that only epithelial cells in both the stomach and small intestine are stained intensely with X-gal. No staining of hematopoietic cells was seen, although immunohistochemistry may not be sensitive enough to identify a few transfected immune cells.

Antibody response against Arah2

To assess the potential utility of oral nanoparticle-mediated gene immunization in modulating the mucosal immune response, we

used a murine model of peanut allergen-induced hypersensitivity. Susceptible strains of mice develop anaphylactic reactions with features similar to those seen in allergic patients²⁶. We induced anaphylaxis by combined oral and intraperitoneal sensitization with crude peanut extract and adjuvants (cholera toxin and alum, respectively), followed by intraperitoneal challenge with recombinant Arah2. In this murine model of peanut hypersensitivity, Arah2 is the dominant anaphylaxis-inducing antigen in mice sensitized with peanut (PN), as demonstrated by Arah2-specific IgE and lymph node T-cell response. The hypersensitivity responses include the induction of specific IgE antibodies and histamine, vascular permeability and active systemic anaphylaxis. Using this model, we first determined whether oral delivery of nanoparticles containing pCMVArah2 was able to induce a substantial immune response. Four weeks after the first immunization, there were substantial differences in the levels of secreted and serum antibodies between nanoparticle-immunized mice and control mice (PBS-fed or naive) or mice immunized with 'naked' DNA (pCMVArah2) (Fig. 3a-c). Mice immunized with pCMVArah2 nanoparticles had increased levels of secretory IgA in fecal extracts, indicating induction of mucosal immune response. In addition, there was a substantial induction of serum anti-Arah2 IgG2a in nanoparticle-treated mice, indicating a T helper-cell type1 response. Mice receiving pCMVArah2 alone ('naked' DNA) without chitosan showed no detectable levels of either fecal IgA or serum IgG2a response at any time points. Because allergic reactions are mainly characterized by an increase in antigen-specific serum IgE levels, we also measured the levels of anti-Arah2 serum IgE before and after sensitization in the immunized and control mice. The increase in serum IgE (level after minus level before) was considerably less salient in the nanoparticle-immunized mice than in the control mice or mice immunized with 'naked' DNA.

Protection of immunized mice against Arah2 challenge

We assessed anaphylactic responses in two separate sets of experiments immediately after intraperitoneal challenge with Arah2 protein in immunized and control mice 1 week after the third sensitization with PN (Fig. 4). Mice receiving sensitization alone without prior immunization or treated with 'naked' DNA (pCMVArah2) showed physical signs of anaphylaxis within 10-20 min of challenge. The symptoms included slight or no activity after prodding, spasmodic labored breathing with retractions and sometimes death. We scored this severity as 3-5. In contrast, considerably less-severe (score of 0-2) and delayed anaphylactic responses were seen after challenge in mice immunized with chitosan-pCMVArah2. As an additional control, we immunized one group of mice ($n = 5$) with chitosan nanoparticles containing the plasmid vector alone (without the Arah2 gene) and sensitized and challenged them. These mice developed anaphy-



Fig. 2 β -galactosidase expression in mouse stomach and small intestine 5 days after oral delivery of DNA nanoparticles. **a-d**, Whole tissue staining for LacZ; only stained sections are shown. Mice were fed high-molecular-weight chitosan-p43LacZ nanospheres at a dose of 50 μ g (**a**) or 100 μ g (**b**) per mouse, PBS (**c**) or 'naked' DNA (p43LacZ; **d**). **e-g**, Histological sections from tissues in **a**. The nuclei were counterstained with nuclear-fast red.

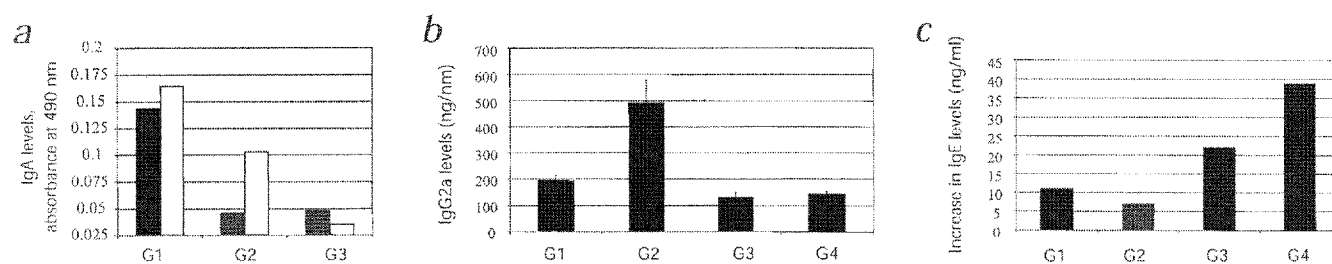


Fig. 3 Antibody responses of immunized and control mice. Mice were immunized with a single dose of chitosan-pArah2 nanoparticles (single dose, G1); with two doses (one week apart) of chitosan-pArah2 nanoparticles (G2); with 'naked' pArah2 (G3); or were not immunized (G4). **a**, Anti-Arah2 IgA response in fecal extracts 3 weeks (■) and 4 weeks (□) after the first immunization, analyzed by Arah2-specific ELISA. Each bar represents values of 'pooled' samples from eight mice (five, for negative controls). **b**, Serum anti-Arah-2 IgG2a levels before sensitization (4 weeks

after first immunization), analyzed by Arah-2 specific ELISA. Each bar represents 'pooled' samples from five mice (two, for non-immunized mice), measured in duplicate. **c**, Increase in serum anti-Arah-2 IgE after sensitization. Serum was collected from the same mice 4 weeks after the first immunization (before sensitization) and after three sensitizations. IgE levels were measured using ELISA. Each bar represents the difference between IgE levels after and before sensitization in 'pooled' serum from five mice/group (two, for non-immunized mice).

axis after challenge with Arah2 similar to that in the control mice (data not shown). All mice recovered in about 2 hours.

In the second set of experiments, one mouse from each immunized and control groups had sudden death that did not seem to be anaphylactic, when challenged after three sensitizations. This phenomenon was not seen in the first set of challenge experiments. The anaphylactic responses of the remaining mice were similar to those in the first set of experiments, and substantial delay and decrease in anaphylactic response were seen in the nanoparticle-immunized mice, as described above.

Plasma histamine levels and vascular leakage

Histamine release from degranulated mast cells and an increase in vascular permeability are two parameters that characterize anaphylaxis. To determine whether the delayed and less-severe PN-induced anaphylactic response seen in nanoparticle-immunized mice was associated with modulation of histamine release, we assayed plasma histamine levels of PN-sensitized, immunized and control mice 10–15 min after Arah2 challenge. The histamine

level in the group immunized with a single dose was substantially lower than that in the control group (Fig. 5a). However, the nanoparticle 'booster' group, which showed partial protection, did not show any substantial reduction in histamine level. To assess changes in the level of vascular permeability upon challenge, we injected Evan's blue dye through the tail veins of mice from the different experimental groups before PN challenge. There was leakage of Evan's blue from peripheral vasculature (skin and feet; Fig. 5b). Mice from groups immunized with chitosan-DNA had much less leakage (blue color in the footpad and skin) than mice from the control and 'naked' DNA-immunized groups.

Discussion

This study demonstrates that chitosan-plasmid DNA nanoparticles delivered orally can modify the immune system in mice and protect against food allergen-induced hypersensitivity. One of the main utilities of such an oral vaccine is its potential to generate mucosal as well as systemic immunity. The mucosal immune system is geared not only to protect against antigenic entry to the systemic immune system but also to be unresponsive to food antigens²⁷. Peanut allergy is a 'mucosal disease' in which the usually unreactive immune system becomes hypersensitive to the ingested protein and generates strong, abnormal anaphylactic reactions. To protect the host against food allergen-induced anaphylaxis, it is therefore ideal to pre-modify the mucosal immune system. Furthermore, the high patient compliance in oral administration, especially in children, gives such a delivery system a considerable advantage. Oral delivery of DNA encapsulated in poly-lactide-co-glycolide microspheres can generate immune response against rotavirus infections²⁶. Oral gene delivery for correcting lactose intolerance in a rat model has also been achieved using an adeno-associated-viral vector²⁹.

Chitosan is a useful oral gene carrier because of its adhesive and transport properties in the gut. Available in oral pill form, chitosan is used as an alternative therapy to reduce dietary fat absorption and cholesterol^{30,31}. Its safety and non-toxicity has been shown in animal models and in humans^{30,32}. Chitosan, when complexed with plasmid DNA, can form stable nanoparticles that can be endocytosed by cells in the gastrointestinal tract. As chitosan is a mucoadhesive polymer^{33–35}, such DNA nanoparticles might adhere to the gastrointestinal epithelia, be transported across the mucosal boundary by M cells and transfect epithelial and/or immune cells in the gut associated lymphoid tissue either directly or through

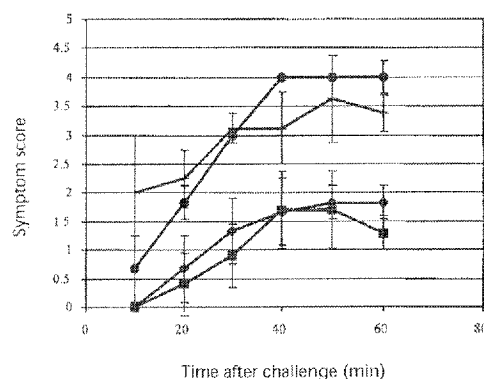
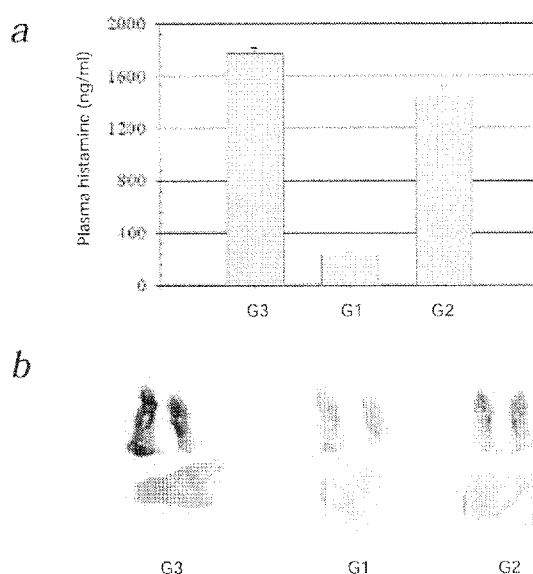


Fig. 4 Anaphylactic response of mice after Arah2 challenge. Mice ($n = 6$) were immunized with a single dose of chitosan-pArah2 nanoparticles (single dose, G1; ◆); with two doses (one week apart) of chitosan-pArah2 nanoparticles (G2; ■); with 'naked' pArah2 (G3; ●); or were not immunized (G4; no symbol). Mice were then sensitized with oral and intraperitoneal doses of crude peanut extract, challenged intraperitoneally with recombinant Arah2 protein, and anaphylaxis was then scored on a scale of 0 to 5. Results represent average anaphylactic response from two separate experiments.

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Fig. 5 Histamine levels and vascular leakage after sensitization and challenge. Mice were immunized with a single dose of chitosan-pArah2 nanoparticles (single dose, G1); with two doses (one week apart) of chitosan-pArah2 nanoparticles (G2); or with 'naked' pArah2 (G3). **a**, Levels of plasma histamine (average of two mice) in mice after sensitizations and challenge, analyzed by competitive ELISA. **b**, Vascular leakage assay. Mice were injected with Evan's blue dye immediately before intraperitoneal challenge with Arah2 and monitored for vascular leakage in the extremities. Blue color (G3) in peripheral tissues indicates dye leakage through the vascular bed because of increased capillary permeability due to anaphylactic or allergic response. Absence of blue color (G1 and G2) indicates absence of anaphylactic vascular leakage.



'antigen transfer', as suggested by the β -galactosidase expression after chitosan-p43LacZ delivery. *In vitro* studies have also shown that chitosan can enhance transcellular and paracellular transport of drugs across intestinal epithelial monolayers²⁴.

The anaphylaxis response in nanoparticle-immunized mice indicates that considerable protection can be achieved against allergen challenge by oral delivery of a single dose of plasmid DNA in particle formulation. Unfortunately, the results of a single 'booster' administration were inconclusive. Further studies will be necessary to determine the effect of multiple doses and kinetics for an optimal vaccination protocol. The level of plasma histamine in the 'booster' group after challenge do not indicate anaphylactic protection. The fact that same degree of protection was still observed indicates that the pathogenesis of allergic anaphylaxis in this murine model may be multifactorial. The lack of a dose-response analysis in this study precludes comparison of this oral immunization approach with other vaccination protocols.

We chose to use a preventive vaccine model instead of a treatment model. This has a better chance of success, especially in an immunopathology like allergy, because the immune system is still naive with respect to the antigen of interest and thus can be modified in a favorable way. In a therapeutic model, the immune system would have been already 'committed' to an anaphylactic pathway and thus it might be much more difficult to re-route the response. However, given that the only treatment of food allergy is complete avoidance of all allergen-containing food products and often involves aggressive emergency treatments, pre-immunization may be a viable therapeutic model. This animal model uses a combination of oral and intraperitoneal sensitizations along with intraperitoneal challenge to generate a hypersensitivity and anaphylactic response that is probably more severe than an oral challenge protocol. Although these results do not mean that protection will be seen in humans, in whom peanut exposure is oral, this model addresses the immunological effects on the most severe symptoms and is therefore important for therapeutic design. Although the optimal dosage, proper booster conditions and efficacy in humans remain to be determined, this study demonstrates the potential of oral DNA vaccination against food allergy by a biocompatible non-viral vector.

Methods

Materials. Chitosan (molecular weight, about 390,000) was a gift from Vanson Chemicals (Redmond, Washington). Arah2, a PCR-amplified Arah2 coding region gene segment with the addition of a Kozak consensus translation codon, was provided by G. Bannon (University of Arkansas). To generate an expression gene construct containing a major peanut allergen gene, we ligated Arah2 into pCR3 expression vector containing CMV promoter/enhancer sequences. The resultant expression construct was called pCMVArah2. The plasmid encoding β -galactosidase (p43LacZ) was a gift from Barry Byrne (University of Florida). Male AKR/J mice 4–5 weeks old were obtained from Jackson Laboratories (Bar Harbor, Maine).

Nanoparticle formulation. Nanoparticles were made by complex coacervation of chitosan and DNA as reported¹⁷. Plasmid (10 μ g) was added to 100 μ l of sodium sulfate and heated to 55 °C. Chitosan (pH 5.7; 0.02% in a 25 mM sodium acetate-acetic acid buffer) was also heated to 55 °C and 100 μ l of chitosan was added to the DNA-sodium sulfate solution while samples were vortexed at high speed for 20 s. Particles were examined immediately by light microscopy and stored at room temperature.

Particle characterization. The particles were characterized by size and zeta potential by photon correlation spectroscopy (dynamic light scattering) using a Zetasizer 3000 (Malvern Instruments, Southborough, Massachusetts) in the 'automatic' analysis mode. The number distribution of particle size, as analyzed by the zetasizer, and the zeta potential (pH 5.7), as measured in an aqueous dip cell, were used for quality control of the nanoparticles. These particles were further characterized by transmission and scanning electron microscopy. For transmission electron microscopy, freshly prepared particles were placed on a glow-discharged carbon grid, allowed to remain there for a few minutes, and then were air-dried. The grid rotary shadowed at 7 degrees with platinum. A Zeiss transmission electron microscope was used to visualize the particles. For scanning electron microscopy, cells were grown on coverslips and incubated with nanospheres for 10 min. After being incubated, they were fixed, dehydrated in graded ethanol, and 'critical-point dried' by replacing ethanol with liquid CO₂ in a pressurized chamber and evaporation of the CO₂ at room temperature. Samples were sputter coated with platinum. The particles on cell surface were then visualized through a scanning electron microscope.

Gene expression. AKR/J mice were fed either chitosan-DNA nanoparticles containing the LacZ gene (p43LacZ, 50 μ g per mice) or 'naked' plasmid DNA (p43LacZ), using animal feeding needles. Five days later, the mice were killed and their stomachs and small intestines were surgically removed. The whole tissues were stained with 4-chloro-5-bromo-3-indolyl- β -galactoside (X-Gal) according to standard protocols. After being stained overnight in a humidified chamber, the tissues were photographed at Pathology Photography (Johns Hopkins Hospital, Baltimore, Maryland). The pictures were scanned into a computer and adjusted for equal brightness and contrast using Adobe Photoshop. The tissues were then frozen in O.C.T. Embedding Medium (Sakura Finetek USA, Torrance, California) and cut into thin sections. The sections were counterstained with nuclear-fast red and visualized under a light microscope equipped with a digital camera. Pictures were adjusted for equal brightness and contrast in Adobe Photoshop.

Immunization. AKR/J mice were immunized orally with various formulations using animal feeding needles (Fisher Scientific) attached to a 1-ml syringe. Each mouse was fed with a dose of about 50 μ g DNA in a volume of

500–900 μ l (depending on the formulations). The anaphylactic protection experiments were repeated with different experimental and control groups. Blood and fecal extracts were collected from immunized and control mice to measure secreted IgA levels in fecal extract, serum IgG2a, increase in serum IgE before and after immunization and release of plasma histamine after antigen challenge.

Collection of serum and fecal extract. Blood was collected at 4 weeks (before sensitization) and 7 weeks (immediately after sensitization but before challenge) through tail veins. After incubating 30 min to 1 h at room temperature, the blood was centrifuged at 4 °C for 25–30 min and serum (supernatant) was collected and stored at –80 °C. Fecal pellets were also collected from the same groups of mice at 3 and 4 weeks after immunization and frozen at –80 °C for further measurements. The supernatant of 100 mg/ml of fecal pellets dissolved in PBS was analyzed (undiluted samples).

Sensitization for peanut allergy. All mice were sensitized three times, 1 week apart, with crude peanut extract. Each sensitization was done over 2 consecutive days; on the first day, by oral administration of 1 mg crude peanut extract with 10 μ g cholera toxin per mouse and on the second day, by intraperitoneal administration of 0.5 μ g extract per mouse with aluminum hydroxide as adjuvant. Each administration volume was 200 μ l per mouse.

ELISA measurements. Serum was collected before and after sensitization and analyzed by ELISA to determine IgE and IgG2a levels. ELISA was done on fecal extracts to measure secretory IgA. Plates were coated with HAS-DNP standards or Arah2 protein (10 μ g/ml) at 4 °C overnight. Plates were blocked for 2 h at 37 °C with 1% BSA-PBST. Serum samples were 'pooled' for each group, added in duplicate (1:5 dilution for IgE and 1:20 for IgG2a) and incubated overnight at 4 °C. Biotinylated anti-mouse IgA, IgE and IgG2a (anti-DNP monoclonal antibodies IgG2a and IgE as standards) were added, followed by streptavidin–HRP and TMB substrate. Absorption at 450 nm was measured in a microplate manager (BioRad, Richmond, California). For histamine measurements, immunized and control mice were challenged with purified Arah2 and after 12–15 min, blood was collected from the optic artery and the plasma was assayed for histamine levels using a competitive ELISA-based histamine kit (Immunotech, Westbrook, Maine).

Challenge with Arah2 and anaphylaxis classification. Non-immunized (sensitized) mice were challenged intraperitoneal with 200 μ g of Arah2 protein. Anaphylactic reactions were graded at different time points according to the following scoring system: 0, no sign of reaction; 1, scratching and rubbing around the nose and head; 2, decreased activity with an increasing respiratory rate, pilar erecti and/or puffing around the eyes; 3, labored respiration and cyanosis around the mouth and tail; 4, slight or no activity after prodding or tremors and convulsion; 5, death.

Vascular leakage study. Anaphylactic vascular leakage was measured using Evan's blue assay. Evan's blue dye (200 μ l) was injected into the tail vein; this was immediately followed by intraperitoneal challenge with 200 μ g Arah2. Mice were monitored for 45 min and killed to record the effect of anaphylactic vascular leakage (blue color in the extremities). Mice that develop anaphylaxis have blue extremities because of vascular leakage; immunized mice that are protected from anaphylaxis are expected to have no (or reduced) blue color. Photographs were taken using a Nikon camera and developed as ektachrome slides. Slides were scanned, enlarged and adjusted for brightness and contrast using Adobe Photoshop.

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